Reactivity of the α-Putrescinylthymine Amino Groups in φW-14 Deoxyribonucleic Acid†

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ABSTRACT: Bacteriophage φW-14 DNA contains the hypermodified pyrimidine α-putrescinylthymine (putThy) [Kropinski, A. M. B., Bose, R. J., & Warren, R. A. J. (1973) *Biochemistry 12*, 151]. The primary amino groups of the putrescinyl side chains react with trinitrobenzenesulfonate (TNBS) but at a much slower rate than the reactive lysine side chains of proteins. Both primary and secondary amino groups in the putrescinyl side chains react with acetic anhydride, but the reaction does not go to completion under the conditions employed. Some of the secondary amino groups react faster with acetic anhydride than do some of the primary amino groups. Acetylation lowers the thermal transition temperature (T_m) of φW-14 DNA, confirming that the unusually high T_m of this DNA is due to the charged putrescinyl groups, but it does not affect the buoyant density. The extent of lowering of the T_m is proportional to the degree of acetylation. Since some of the secondary amino groups are acetylated before some of the primary amino groups, the effect of acetylation on the T_m suggests that both the primary and secondary amino groups can neutralize negative charge repulsion in φW-14 DNA. Only the putThy residues in the DNA are modified by TNBS and acetic anhydride.

The deoxyribonucleic acid (DNA)[1] of bacteriophage φW-14 contains 51 mol % G+C (Maltman et al., 1980). It is unusual because half the thymine residues are replaced with α-putrescinylthymine (putThy) (Kropinski et al., 1973). The presence of this hypermodified pyrimidine affects markedly the physical properties of φW-14 DNA (Kropinski et al., 1973); it allows for the very tight packing of the DNA within the virion head (D. G. Scraba et al., unpublished experiments); it probably accounts for the alkali lability of the DNA (Lewis et al., 1975).

The putrescinyl groups are analogous to lysyl side chains on proteins, except that they have both primary amine character and secondary amine character. In theory, they should be modifiable with reagents used to modify the lysyl side chains on proteins (Means & Feeney, 1971). Such modification would allow, for example, evaluation of the contribution of the positive charges on the putrescinyl side chains to the physical properties of φW-14 DNA, the preparation of derivatives for use in studies on the solution conformation of the DNA, and covalent attachment of the DNA to inert supports for use in affinity chromatography.

Two reagents have been used initially to examine the reactivity of the putThy amino groups in φW-14 DNA: 2,4,6-trinitrobenzenesulfonate (TNBS), a reagent introduced for the colorimetric determination of amino acids (Okuyama & Satake, 1960) and used subsequently for the determination of amino groups in proteins (Fields, 1971), and acetic anhydride.

Analyses of the products formed in these reactions have been facilitated by the differential labeling of the bases in φW-14 DNA. [2-^{3}H]Adenine labels the purines, [6-^{3}H]uracil labels all the pyrimidines, [5-^{3}H]uracil labels the cytosines, and [2,3-^{3}H]ornithine labels putThy only (Quail et al., 1976). This is important because adenine, guanine, and cytosine have amino groups that, even though hydrogen bonded within the helix, might also react with the modifying reagents. The results show that under the conditions employed, only the putThy residues react significantly.

Materials and Methods

Preparation of φW-14 DNA. φW-14 virions were purifed from lysates as described previously (Kropinski et al., 1973). The particles were suspended in TNE (0.01 M Tris-HCl, 0.15 M NaCl, and 0.01 M EDTA, pH 7.4), sufficient Pronase solution (20 mg mL^{-1} in TNE, self-digested for 30 min at 37 °C) was added to give a final concentration of 2 mg mL^{-1}, followed by sufficient 10% $NaDodSO_4$ (sodium dodecyl sulfate) solution to give a final concentration of 0.5%, and the mixture was incubated at 37 °C for 18 h. The DNA solution was extracted twice with an equal volume of redistilled phenol, preequilibrated with TNE. Residual phenol was removed by washing the solution several times with ether. The final solution was stored at 4 °C over chloroform. Before use, the DNA was dialyzed twice against at least 100 volumes of 0.15 M NaCl. The final DNA concentrations ranged from 250 to 500 μg mL^{-1}.

Reaction with TNBS. The extent of reaction of the DNAs with TNBS at pH 9.5 and room temperature was determined by measuring the absorbance of the sulfite complexes of the TNP amino groups at pH 6.0 (Fields, 1972).

Reaction with Acetic Anhydride. Initially, DNAs were reacted with acetic anhydride in 0.1 M triethanolamine hydrochloride (TEA), pH 8.5, the pH being maintained by the addition of 4 N NaOH [see Stuart & Khorana (1964)]. However, φW-14 is unusually alkali labile (Lewis et al., 1975) so that even brief exposure to strong alkali might produce spurious results. Subsequently, it was found that if the TEA concentration was increased to 0.5 M, the pH did not drop significantly during the course of the reaction. A 15-mL sample of DNA in 0.15 M NaCl was mixed with 5 mL of 2 M TEA, pH 8.5, in a 30-mL beaker. The solution was stirred magnetically at room temperature and 100 μL of acetic anhydride added at 0 time, after 6 min, and after 12 min. DNA

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[1] Abbreviations: DNA, deoxyribonucleic acid; putThy, α-putrescinylthymine; TNBS, 2,4,6-trinitrobenzenesulfonate; TEA, triethanolamine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; HF, hydrofluoric acid; T_m, thermal dissociation temperature; Tnp, 2,4,6-trinitrophenyl; putdThd, α-putrescinylthymidine; putdTMP, α-putrescinylthymidine monophosphate; dCyd, deoxycytidine; dThd, thymidine; putTnpdThd, α-putrescinyl(δ-trinitrophenyl)thymidine; $NaDodSO_4$, sodium dodecyl sulfate.

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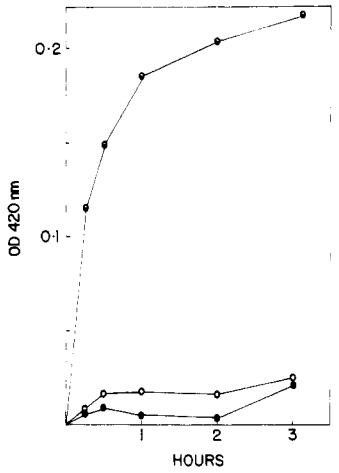


FIGURE 1: Reaction of DNAs with TNBS. (◒) φW-14 DNA; (○) acetylated φW-14 DNA; (●) calf thymus DNA.

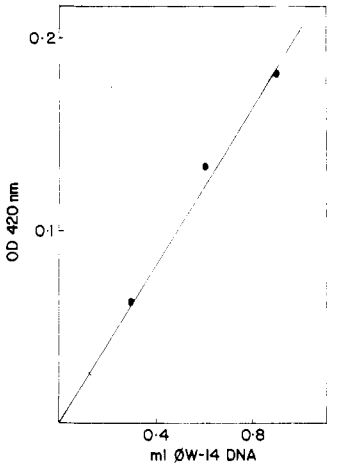


FIGURE 2: Color intensity as a function of DNA concentration for the reaction of φW-14 DNA with TNBS. Reaction time was 3 h.

to be used for the examination of reaction products was allowed to react for 30 min, the reaction stopped by adding excess 1 M ethanolamine, pH 8.5, and the DNA precipitated with ethanol. The kinetics of acetylation were followed in the same way except that samples of 4 mL were transferred at intervals to polypropylene tubes containing 100 μL of 1 M ethanolamine, pH 8.5. After the last sample was taken, 8 mL of 95% ethanol was added to precipitate the DNA. The DNA was washed twice with 95% ethanol and once with acetone and then allowed to air-dry. The samples were hydrolyzed with hydrofluoric acid (HF) to release the pyrimidine nucleosides and the purine bases (Walker & Mandel, 1978).

Analysis of the Products. DNA labeled with the appropriate radioactive precursor was hydrolyzed with HF (see above) or with nuclease S1 and snake venom phosphodiesterase to release the mononucleotides (Maltman et al., 1981). Products were separated by thin-layer chromatography on sheets of unmodified cellulose (Eastman 6064 chromogram, without fluorescent indicator) (Warren, 1981; Maltman et al., 1981). The products were detected by fluorography (Randerath, 1969), and their radioactivity was determined (Warren, 1981).

Other Methods. Thermal dissociation temperatures (T_ms) were determined with a Gilford 2527 thermoprogrammer and Gilford 250 spectrophotometer at a rate of temperature increase of 1 °C min^{-1}. Buoyant densities of DNAs were determined by isopycnic density gradient centrifugation in CsCl (Maltman et al., 1980).

Chemicals and Enzymes. Calf thymus DNA and trinitrobenzenesulfonic acid were from Sigma Chemical Co., St. Louis, MO; the latter was recrystallized before use (Fields, 1971). Nuclease S1 was from Miles Laboratories, Elkhart, IN. Snake venom phosphodiesterase was from Millipore, Montreal, P.Q., Canada. [5-^{3}H]Uracil, [6-^{3}H]uracil, L-[2,3-^{3}H]ornithine, [2-^{3}H]adenine, [^{3}H]acetic anhydride, and [^{32}P]orthophosphate were from New England Nuclear, Montreal, P.Q., Canada.

Results

Reaction of DNAs with TNBS. Calf thymus DNA reacted only slightly with TNBS in 3 h (Figure 1). φW-14 DNA, however, reacted strongly with this reagent (Figure 1), and the extent of reaction was proportional to the amount of DNA added (Figure 2).

The trinitrophenyl (Tnp) derivatives of amino acids are stable to hydrolysis in strong acids at elevated temperatures (Okuyama & Satake, 1960). Therefore, the products of the reaction of φW-14 DNA with TNBS for 3 h were determined after hydrolysis of the DNA with HF. A single yellow product was detected visually. When the DNA was labeled with [^{3}H]ornithine, 97% of the radioactivity applied to the chromatogram comigrated with the yellow product, and the remainder stayed near the origin and was presumed to be unreacted α-putrescinylthymidine (putdThd) (data not shown). Since TNBS reacts at a negligible rate with secondary amines (Means et al., 1972), the product was assumed to be α-putrescinyl(δ-trinitrophenyl)deoxythymidine (putTnpdThd).

TNBS has been reported to trinitrophenylate some 75% of the 2-amino groups in the guanines of double-stranded DNA in 24 h at 37 °C but not to react with the amino groups on the cytosines and adenines (Azegami & Iwai, 1964). φW-14 DNA labeled with [2-^{3}H]adenine was reacted with TNBS for 3 h. After hydrolysis with HF, adenine and guanine (in a 1:1 ratio) were the only radioactive products (data not shown).

Acetylation of φW-14 DNA. Acetic anhydride reacts readily with both primary and secondary amines, so putThy could be mono- and/or diacetylated by reacting φW-14 DNA with this reagent. Before the kinetics of acetylation were determined, it was necessary to identify the products of the reaction. φW-14 DNA preparations labeled with a variety of radioactive precursors were reacted with unlabeled acetic anhydride; an unlabeled preparation was reacted with labeled reagent. After treatment, half of each preparation was hydrolyzed with HF and half with nuclease S1/snake venom phosphodiesterase. Enzymatic digestion was used in case any of the derivatives formed were labile in HF.

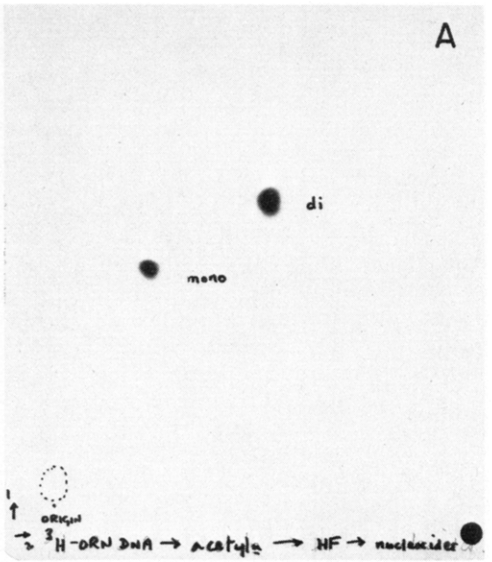
HF hydrolysis or enzymatic digestion of acetylated [^{3}H]-ornithine-labeled DNA released two labeled products, one of which contained more radioactivity than the other (Figure 3 and Table I). The mobilities of the products in the two-dimensional systems used to separate them suggested that the more heavily labeled product was the diacetylated derivative. This was confirmed by electrophoretic analysis of the mononucleotides. At pH 7.5, the diacetylated and monoacetylated derivatives of α-putrescinylthymidine monophosphate (putdTMP) should have net charges of 2− and 1−, respectively. The more heavily labeled product migrated 9.5 cm from the origin, and the other product 6.0 cm, during electrophoresis on a cellulose thin-layer sheet at this pH.

After acetylation of unlabeled DNA with [^{3}H]acetic anhydride, again only two labeled products were obtained, chromatographically similar to, and in the same proportion as, the products obtained from ornithine-labeled DNA (Table I). The products from the two preparations were shown to

Table I: Acetylation of ϕW-14 DNA

source of label in DNA	products measured	radioactivity (cpm) recovered in: monoacetyl-putThy	diacetyl-putThy	putThy	diacetyl in acetylated products (%)
[^{3}H]ornithine	deoxymononucleotides (i)[a]	14396	36261	173	71
	deoxymononucleotides (ii)	20172	38148	145	65
	deoxynucleosides (i)	5926	11596	73	66
	deoxynucleosides (ii)	1941	3473	32	64
[^{3}H]acetic anhydride	deoxynucleosides (iii)	3584	6491		48
	deoxynucleosides (iv)	1563	3914		68
[6-^{3}H]uracil	deoxymononucleotides (v)[b]	3261	3285	63	50
	deoxynucleosides (v)	20096	50449	1095	72

[a] (i) etc. refers to DNA preparations. [b] In this digest, dCMP gave 16 326 cpm and dTMP 8735 cpm.



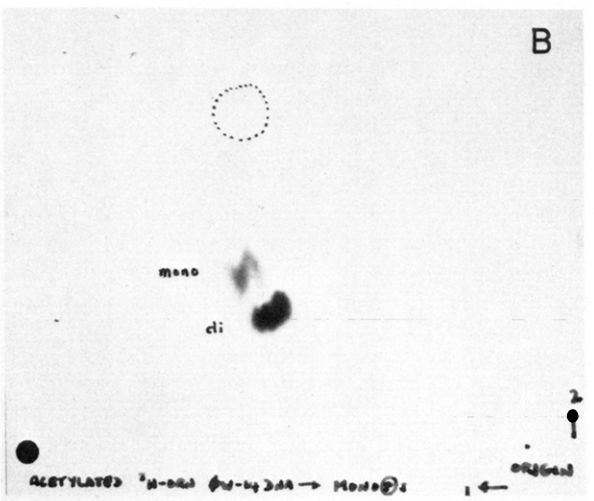


FIGURE 3: Fluorogram of the products obtained from acetylated [^{3}H]ornithine-labeled ϕW-14 DNA. (A) Nucleosides; (B) nucleotides. The dotted circles mark the usual positions of the putThy derivatives.

be identical by cochromatography (data not shown).

These results suggested that only the putThy residues were being acetylated under the conditions employed. This was substantiated by examining the products obtained after acetylation of ϕW-14 DNA labeled with different radioactive precursors. Both before acetylation and after acetylation, [5-^{3}H]uracil-labeled DNA yielded deoxycytidine (dCyd) as the only radioactive product, and [2-^{3}H]adenine-labeled DNA yielded adenine and guanine in a 1:1 ratio as the only radioactive products (data not shown). [6-^{3}H]Uracil-labeled DNA yielded four radioactive products: dCyd and thymidine (dThd)

Table II: Kinetics of Acetylation of ϕW-14 DNA

DNA labeled with	reaction time (min)	cpm in: putThy	monoacetyl-putThy	diacetyl-putThy	total
[^{3}H]-ornithine	2.5	733 (12)[a]	4261 (69)	1152 (19)	6146
	5	251 (4)	4261 (69)	1913 (30)	6425
	10	89 (1)	2949 (46)	3314 (52)	6352
	20	90 (1)	1941 (35)	3473 (63)	5504
[^{3}H]acetic anhydride	2.5		6208 (80)	1539[b] (20)	7747
	5		7273 (69)	3268[b] (31)	10541
	10		6088 (50)	5995[b] (50)	12083
	20		3914 (33)	7817[b] (67)	11731

[a] Figures in parentheses are the percentages of the total cpm recovered. [b] These are half the actual cpm because the specific activity of this diacetyl is twice that of the monoacetyl derivative.

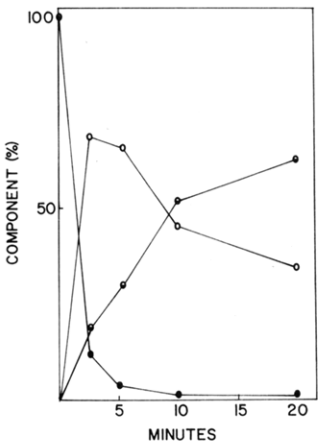


FIGURE 4: Kinetics of acetylation of [^{3}H]ornithine-labeled ϕW-14 DNA. (●) putThy; (○) monoacetyl-putThy; (◒) diacetyl-putThy. Plotted from the data in Table II.

in a 1:1 ratio and the mono- and diacetylated putdThds in the usual proportions (Table I).

Kinetics of Acetylation. The kinetics of acetylation were determined by using ornithine-labeled DNA. All of the putThy residues reacted within 10 min, almost 90% of them within 2.5 min; the monoacetylated derivative was formed faster than the diacetylated derivative (Table II and Figure 4). The same kinetics were observed with unlabeled DNA and [^{3}H]acetic anhydride (Table II).

Position of Acetylation in Monoacetylated putThy. Since TNBS reacts at a negligible rate with secondary amines (Okuyama & Satake, 1960), it could be used to determine the position of acetylation in monoacetyl-putThy. A sample of ornithine-labeled DNA was treated with acetic anhydride for 2.5 min, the reaction stopped with an excess of ethanolamine, and the DNA dialyzed against 0.15 M NaCl. Half of the preparation was hydrolyzed with HF, and the remainder was

Table III: Analysis of Nucleosides after Acetylation of φW-14 DNA with [^{3}H]Acetic Anhydride

time for acetylation (min)	further treatment	cpm in monoacetyl-putThy	cpm in diacetyl-putThy[a]	cpm in Tnp derivative	total cpm
2.5	nil	10 130 (62)[b]	6 210 (38)		16 330
	trinitrophenylation	1 850 (14)	4 380 (32)	7300 (54)	13 530
20.0	nil	13 000 (47)	14 840 (53)		27 840
	trinitrophenylation	1 880 (15)	5 980 (47)	5060 (39)	12 820

[a] These are half the actual cpm because the specific activity of the diacetyl is twice that of the monoacetyl derivative. [b] Figures in parentheses are the percentages of the total cpm recovered.

Table IV: T_ms of Acetylated DNAs[a]

DNA	acetylation	preparation	T_m[b] (°C)	indicated mol % G+C
φW-14	−		85.3	71[c]
	+	1	72.5	45
	+	2	72.2	45
	+	3	76.0	52
calf thymus	−		69.5	
	+	1	68.5	
	+	2	68.7	

[a] DNAs were acetylated for 20 min. [b] In 0.015 M NaCl. [c] The actual mol % G+C for φW-14 DNA is 51.

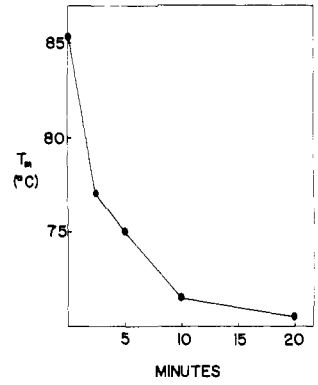


FIGURE 5: T_m of φW-14 DNA as a function of the time of acetylation.

reacted with TNBS for 3 h and then hydrolyzed with HF. Analysis of the products showed that the monoacetyl-putThy was a mixture of products. After acetylation of unlabeled DNA with [^{3}H]acetic anhydride for 2.5 min, some 80% of the monoacetyl derivative was acetylated on the secondary amino group; after acetylation for 20 min, some 73% of the monoacetyl derivative was acetylated on the secondary amino group (Table III). In other words, many of the secondary amino groups were reacting before the primary amino groups.

Effect of Acetylation on the Physical Properties of φW-14 DNA. Acetylation lowered the T_m of φW-14 DNA, but this treatment did not alter the T_m of calf thymus DNA. Acetylation did not alter the buoyant density of φW-14 DNA (Table IV). Since acetylation seemed to occur at the secondary amino groups before the primary amino groups, the T_m of the DNA was determined as a function of the time of acetylation in an attempt to determine which of the amino groups was most influential in raising the T_m of φW-14 DNA. The rate of lowering of the T_m was roughly proportional to the rate of acetylation of the DNA with [^{3}H]acetic anhydride (Figures 4 and 5).

Discussion

It is clear that the putrescinyl amino groups in φW-14 DNA can be modified selectively and that the DNA remains double helical after modification. Hydrogen bonding within the double helix probably protects the amino groups on adenine, guanine, and cytosine from the modifying reagents (Stuart & Khorana, 1964; Kochetov & Budowsky, 1969). The mild pHs (8.5–9.5) used here would ensure that the hydrogen bonding was maintained during the reactions. Although the guanine residues in double-helical DNA can react during prolonged exposure to TNBS (Azegami & Iwai, 1964), there was negligible modification of the guanine residues in φW-14 DNA during the 3-h reaction time used in the present work. That the putrescinyl groups were responsible for the reaction of φW-14 DNA with modifying reagents was shown also by the negligible reactions of calf thymus DNA and acetylated φW-14 DNA with TNBS. The reaction with acetylated φW-14 DNA probably occurred at nonacetylated primary amino groups in the putrescinyl side chains (see later).

TNBS reacts much less readily with φW-14 DNA than it does with proteins (Figure 1; Fields, 1971). DNA is a polyanion, and the rate of reaction of TNBS is decreased by electrostatic interactions of the reagent with negatively charged neighboring groups (Means et al., 1972). The reaction is sensitive also to steric hindrance (Fields, 1971; Means et al., 1972). The reaction profile for φW-14 DNA (Figure 1) suggests that some of the purtrescinyl amino groups react slower than others. This agrees with some of the secondary amino groups reacting faster with acetic anhydride than some of the primary amino groups. If there are putThy groups in clusters, the grouping together of the charged amino groups could allow a rapid initial reaction with some of them because of the localized reduction in net negative charge. Subsequent reactions would be slowed by electrostatic repulsion of the TNBS and by steric hindrance from the neighboring Tnp groups.

Sulfite complexes of Tnp amino groups may interact with each other or with aromatic side chains in proteins to decrease the molar absorbances of these groups (Fields, 1972). The molar extinction coefficient for Tnp-putThy in φW-14 DNA is about 9000 M^{-1} cm^{-1}. This is significantly lower than the values obtained for α- and ε-Tnp-lysine (Fields, 1972); it probably reflects interaction of the Tnp amino groups with each other and, perhaps, with the DNA bases. It may also reflect the shielding of the Tnp amino groups from the sulfite ion by the phosphate backbone.

That some of the secondary amino groups react with acetic anhydride faster than some of the primary amino groups is at first sight surprising. In the B conformation for DNA, the secondary amino groups would lie to one side of the major groove and would be more sterically hindered than the primary amino groups. However, the primary amino groups may lie closer to negatively charged phosphates than do the secondary amino groups. The proximity of charged groups can affect the reactivity of functional groups toward chemical reagents. The N-terminal lysine of porcine elastase is involved in an electrostatic interaction with an internal aspartate, and it has a lower reactivity than expected toward acetic anhydride (Kaplan et al., 1971). The amino groups in free histones are

more reactive toward acetic anhydride than they are in chromatin (Malchy, 1977). Thus, the ionic interaction of the primary amino groups with the negatively charged phosphates, coupled with the fact that secondary amines react with acetic anhydride faster than primary amines (Malchy, 1977), could explain this anomaly. However, interaction of the amino groups of polylysine with calf thymus DNA apparently does not block modification by acetic anhydride at pH 8.3–9.0 (Tack & Simpson, 1979). The kinetics of acetylation were not followed. It is possible that after a certain level of acetylation, the complex dissociates and the remaining acetylation occurs on polylysine molecules free in solution (Tack & Simpson, 1979).

The reactivity of the putrescinyl amino groups with acetic anhydride has been used to show that the putrescinyl side chains are crucial to at least one biological property of ϕW-14 DNA: the native DNA is a potent inhibitor of transformation in *Bacillus subtilis* (Lopez et al., 1980); acetylation blocks this effect (Lopez et al., 1982). Acetylation and trinitrophenylation have shown also that the putrescinyl amino groups are not further modified in ϕW-14 DNA. This agrees with the earlier suggestion (Kropinski et al., 1973) that the T_m of this DNA is unusually high because putThy residues reduce negative charge repulsion, and this is supported by lowering of the T_m by acetylation.

Acetylation can lower the T_m below that expected for a DNA of 51 mol % G+C (Table III), suggesting that the helix is being destabilized. Since the DNA double helix can accommodate quite large uncharged substituents without destabilization (glucosylated T4 DNA has the T_m expected for a DNA of 34 mol % G+C), it is not clear why acetylation destabilizes ϕW-14 DNA.

It is probable that both the nature of the side chain and its positive charges affect the buoyant density of ϕW-14 DNA (Kropinski et al., 1973), since positive charges could exclude cesium ions. However, acetylation did not affect the buoyant density, so it appears that the increased cesium ion binding is counterbalanced by the acetyl groups serving to decrease the density.

The kinetics of acetylation and the effect of acetylation on the T_m suggest that the solution conformation of ϕW-14 DNA is such that both the primary and secondary amino groups of the putrescinyl side chains can reduce repulsion between the negatively charged phosphates. It is not clear if these amino groups must interact ionically with the phosphates to produce their effect. However, the conformation must be such that the greater reactivity of the secondary, relative to the primary, amino groups (Malchy, 1977) is not reduced by steric effects.

We are determining the kinetics of acetylation of ϕW-14 as a function of pH and salt concentration to see what factors influence the reactivities of these amino groups.

Acknowledgments

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